10/070982

Patent Office
Canberra

AU 00/01083

I, KAY WARD, ACTING MANAGER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PQ 2728 for a patent by CSL LIMITED, THE COUNCIL OF THE QUEENSLAND INSTITUTE OF MEDICAL RESEARCH, WALTER AND ELIZA HALL INSTITUTE OF MEDICAL RESEARCH, THE UNIVERSITY OF MELBOURNE and COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION filed on 09 September 1999.

WITNESS my hand this
Sixth day of October 2000

KAY WARD
ACTING MANAGER EXAMINATION
SUPPORT AND SALES

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH RULE 17.1(a) OR (b)

AUSTRALIA

Patents Act 1990

CSL Limited
The Council of the Queensland Institute of Medical Research
The Walter and Eliza Hall Institute of Medical Research
The University of Melbourne
Commonwealth Scientific and Industrial Research Organisation

PROVISIONAL SPECIFICATION

Invention Title:

Dendritic Cell Membrane Protein FIRE

The invention is described in the following statement:

Dendritic Cell Membrane Protein FIRE

FIELD OF THE INVENTION

The present invention relates to a protein (designated FIRE) which is preferentially expressed in dendritic cells stimulatory to T cells and to nucleic acid sequences encoding this protein. The invention also relates to uses of the protein and nucleic acids.

BACKGROUND OF THE INVENTION

Dendritic cells (DC) are antigen presenting leukocytes which play a critical role in the initiation of immune responses. To stimulate naive T lymphocytes, which is an essential step in generating the immunological memory required for effective vaccination, it is crucial for antigen to be presented by DC. Over the last 8 years techniques have been developed to purify DC populations and lineages from mouse lymphoid organs. This DC purification protocol involves density centrifugation, depletion of contaminating cells with a monoclonal antibody cocktail and magnetic beads, and finally Fluorescent Activated Cell Sorting. Using an original version of this purification protocol, two DC populations in mouse spleen were identified, which are defined by their expression of two cell surface proteins: a lymphoid-lineage related $CD8^+Mac\text{-}1^-$ DC and a myeloid-lineage related $CD8^-Mac\text{-}1^+$ DC. These two populations differ in their interactions with T lymphocytes. Although the two DC populations displayed equivalent ability to stimulate T cells into cell cycle, they differed in their ability to induce the production of cytokines such as IL-2 and IL-3, which are critical for the induction of an effective immune response (1-5). The myeloid related $CD8^-Mac\text{-}1^+$DC are much more efficient in cytokine induction than the lymphoid related $CD8^+Mac\text{-}1^-$ DC. Other differences between the DC are the levels of cytokines they themselves produce (such as IL-12) which, potentially, could regulate the nature as well as the quality of cytokines they induce in the activated T cells. The molecular mechanisms which underpin these differences in interactions between the two DC populations and T lymphocytes are unknown. Presumably myeloid DC differentially express molecules which enable them to stimulate T lymphocytes to produce certain cytokines more efficiently than do lymphoid DC, or alternatively lymphoid DC differentially express molecules which inhibit the stimulation of T

lymphocytes. To address this question the present inventors compared gene expression in the two DC populations using the technique of Representational Difference Analyses (RDA)(6).

Briefly, RDA identifies differential gene expression between two given cell types by using successive rounds of a combination of PCR and subtractive hybridisation which generates DNA fragments of putatively differentially expressed genes (6).

A full length clone encoding a novel gene was obtained using conventional molecular biological techniques, which used the original RDA generated fragments. This sequence was designated "FIRE" and encodes a 723 amino acid protein. Analyses of the FIRE sequence shows that it is a novel member of the recently described EGF/TM7 superfamily (7).

SUMMARY OF THE INVENTION

In a first aspect the present invention consists in a polypeptide, the polypeptide having an amino acid sequence as set out in Figure 3, or a sequence having at least 50% identity thereto, or a functional fragment thereof.

The term "functional fragment" as used herein is intended to cover fragments of the polypeptide which retain at least 10% of the biological activity of the complete polypeptide. In particular this term is used to encompass fragments which show immunological cross-reactivity with the entire polypeptide, eg ligands which react with the fragment also react with the complete polypeptide.

In a preferred embodiment the polypeptide has a sequence of at least 70%, more preferably at least 80% and most preferably at least 90% identity with the sequence shown in Figure 3.

In a second aspect the present invention consists in a ligand, the ligand being directed against the polypeptide of the first aspect of the present invention.

In a preferred embodiment the ligand is an antibody or the binding portion thereof.

In a third aspect the present invention consists in a nucleic acid molecule, the nucleic acid molecule encoding the polypeptide of the first aspect of the present invention or the binding region of the ligand of the second aspect of the present invention.

In a preferred embodiment of this aspect of the present invention the nucleic acid molecule has a sequence as set out in Figure 3 or a sequence having at least 60% identity thereto, or a sequence which hybridises thereto under stringent conditions.

In a preferred embodiment the nucleic acid molecule has a sequence of at least 70%, more preferably at least 80% and most preferably at least 90% identity with the sequence shown in Figure 3.

In a fourth aspect the present invention consists in a composition for use in raising an immune response in a subject, the composition comprising a ligand of the second aspect of the present invention and an antigen and optionally a carrier and/or adjuvant.

In a preferred embodiment the antigen is conjugated to the ligand.

In a fifth aspect the present invention consists in a composition for use in raising an immune response in a subject, the composition comprising a DNA molecule and a carrier, the DNA molecule comprising a first sequence encoding a ligand of the second aspect of the present invention and a second sequence encoding an antigen.

In a sixth aspect the present invention consists in a method of screening compounds for immunological regulatory activity, the method comprising reacting the compound with the polypeptide of the first aspect of the invention and measuring interaction between the compound and the polypeptide.

DETAILED DESCRIPTION OF THE INVENTION

In order that the nature of the present invention may be more clearly understood preferred forms thereof will now be described with reference to the following non-limiting Examples.

General Molecular Biology

Unless otherwise indicated, the recombinant DNA techniques utilized in the present invention are standard procedures, well known to those skilled in the art. Such techniques are described and explained throughout the literature in sources such as, J. Perbal, A Practical Guide to Molecular Cloning, John Wiley and Sons (1984), J. Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory Press (1989), T.A.

Brown (editor), Essential Molecular Biology: A Practical Approach, Volumes 1 and 2, IRL Press (1991), D.M. Glover and B.D. Hames (editors), DNA Cloning: A Practical Approach, Volumes 1-4, IRL Press (1995 and 1996), and F.M. Ausubel et al. (Editors), Current Protocols in Molecular Biology, Greene Pub. Associates and Wiley-Interscience (1988, including all updates until present) and are incorporated herein by reference.

Protein Variants

Amino acid sequence variants can be prepared by introducing appropriate nucleotide changes into DNA, or by *in vitro* synthesis of the desired polypeptide. Such variants include, for example, deletions, insertions or substitutions of residues within the amino acid sequence. A combination of deletion, insertion and substitution can be made to arrive at the final construct, provided that the final protein product possesses the desired characteristics. The amino acid changes also may alter post-translational processes such as changing the number or position of glycosylation sites, altering the membrane anchoring characteristics, altering the intra-cellular location by inserting, deleting or otherwise affecting the transmembrane sequences of the native protein, or modifying its susceptibility to proteolytic cleavage.

In designing amino acid sequence variants, the location of the mutation site and the nature of the mutation will depend on characteristic(s) to be modified. The sites for mutation can be modified individually or in series, e.g., by (1) substituting first with conservative amino acid choices and then with more radical selections depending upon the results achieved, (2) deleting the target residue, or (3) inserting residues of other ligands adjacent to the located site.

A useful method for identification of residues or regions for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (*Science* (1989) **244**: 1081-1085*)*. Here, a residue or group of target residues are identified (e.g., charged residues such as Arg. Asp, His, Lys, and Glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains demonstrating functional sensitivity to the

substitutions then are refined by introducing further or other variants. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation *per se* need not be predetermined. For example, to optimise the performance of a mutation at a given site, alanine scanning or random mutagenesis may be conducted at the target codon or region and the expressed variants are screened for the optimal combination of desired activity.

There are two principal variables in the construction of amino acid sequence variants; the location of the mutation site and the nature of the mutation. These may represent naturally occurring alleles or predetermined mutant forms made by mutating the DNA either to arrive at an allele or a variant not found in nature. In general, the location and nature of the mutation chosen will depend upon the characteristic to be modified.

Amino acid sequence deletions generally range from about 1 to 30 residues, more preferably about 1 to 10 residues and typically about 1 to 5 contiguous residues.

Amino acid sequence insertions include amino and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Other insertional variants include the fusion of the N- or C-terminus of the proteins to an immunogenic polypeptide e.g. bacterial polypeptides such as betalactamase or an enzyme encoded by the *E. coli trp* locus, or yeast protein, bovine serum albumin, and chemotactic polypeptides. C-terminal fusions with proteins having a long half-life such as immunoglobulin constant regions (or other immunoglobulin regions), albumin, or ferritin, are included.

Another group of variants are amino acid substitution variants. These variants have at least one amino acid residue in the protein molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include sites identified as the active site(s). Other sites of interest are those in which particular residues obtained from various species are identical. These positions may be important for biological activity. These sites, especially those falling within a sequence of at least three other identically conserved sites, are substituted in a relatively conservative manner. Such conservative substitutions are shown in Table 1 under the heading of "preferred substitutions". If such substitutions result in

a change in biological activity, then more substantial changes, denominated exemplary substitutions in Table 1, or as further described below in reference to amino acid classes, are introduced and the products screened.

TABLE 1

Original Residue	**Exemplary Substitutions**	**Preferred Substitutions**
Ala (A)	val; leu; ile	val
Arg (R)	lys; gln; asn	lys
Asn (N)	gln; his; lys; arg	gln
Asp (D)	glu	glu
Cys (C)	ser	ser
Gln (Q)	asn	asn
Glu (E)	asp	asp
Gly (G)	pro	pro
His (H)	asn; gln; lys; arg	arg
Ile (I)	leu; val; met; ala; phe norleucine	leu
Leu (L)	norleucine, ile; val; met; ala; phe	ile
Lys (K)	arg; gln; asn	arg
Met (M)	leu; phe; ile;	leu
Phe (F)	leu; val; ile; ala	leu
Pro (P)	gly	gly
Ser (S)	thr	thr
Thr (T	ser	ser
Trp (W)	tyr	tyr
Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe; ala; norleucine	leu

Mutants, Variants and Homology - Proteins

Mutant polypeptides will possess one or more mutations which are deletions, insertions, or substitutions of amino acid residues. Mutants can be either naturally occurring (that is to say, purified or isolated from a natural source) or synthetic (for example, by performing site-directed mutagensis on the encoding DNA). It is thus apparent that polypeptides of the invention can be either naturally occurring or recombinant (that is to say prepared using recombinant DNA techniques).

An allelic variant will be a variant that is naturally occurring within an individual organism.

Protein sequences are homologous if they are related by divergence from a common ancestor. Consequently, a species homologue of the protein will be the equivalent protein which occurs naturally in another species. Within any one species a homologue may exist as numerous allelic variants, and these will be considered homologues of the protein. Allelic variants and species homologues can be obtained by following standard techniques known to those skilled in the art. Preferred species homologues include those obtained from representatives of the same Phylum, more preferably the same Class and even more preferably the same Order.

A protein at least 50% identical, as determined by methods well known to those skilled in the art (for example, the method described by Smith, T.F. and Waterman, M.S. (1981) Ad. Appl Math., 2: 482-489, or Needleman, S.B. and Wunsch, C.D. (1970) J. Mol. Biol., 48: 443-453), to that of the present invention are included in the invention, as are proteins at least 70% or 80% and more preferably at least 90% identical to the protein of the present invention. This will generally be over a region of at least 20, preferably at least 30, contiguous amino acids.

Mutants, Variants and Homology - Nucleic Acids

Mutant polynucleotides will possess one or more mutations which are deletions, insertions, or substitutions of nucleotide residues. Mutants can be either naturally occurring (that is to say, isolated from a natural source) or synthetic (for example, by performing site-directed mutagensis on the DNA). It is thus apparent that polynucleotides of the invention can be either

naturally occurring or recombinant (that is to say prepared using recombinant DNA techniques).

An allelic variant will be a variant that is naturally occurring within an individual organism.

Nucleotide sequences are homologous if they are related by divergence from a common ancestor. Consequently, a species homologue of the polynucleotide will be the equivalent polynucleotide which occurs naturally in another species. Within any one species a homologue may exist as numerous allelic variants, and these will be considered homologues of the polynucleotide. Allelic variants and species homologues can be obtained by following standard techniques known to those skilled in the art. Preferred species homologues include those obtained from representatives of the same Phylum, more preferably the same Class and even more preferably the same Order.

A polynucleotide at least 70% identical, as determined by methods well known to those skilled in the art (for example, the method described by Smith, T.F. and Waterman, M.S. (1981) Ad. Appl Math., 2: 482-489, or Needleman, S.B. and Wunsch, C.D. (1970) J. Mol. Biol., 48: 443-453), to that of the present invention are included in the invention, as are proteins at least 80% or 90% and more preferably at least 95% identical to the polynucleotide of the present invention. This will generally be over a region of at least 60, preferably at least 90, contiguous nucleotide residues.

Antibody Production

Antibodies, either polyclonal or monoclonal, which are specific for a protein of the present invention can be produced by a person skilled in the art using standard techniques such as, but not limited to, those described by Harlow et al. Antibodies: A Laboratory Manual, Cold Springs Harbor Laboratory Press (1988), and D. Catty (editor), Antibodies: A Practical Approach, IRL Press (1988).

Various procedures known in the art may be used for the production of polyclonal antibodies to epitopes of a protein. For the production of polyclonal antibodies, a number of host animals are acceptable for the generation of antibodies by immunization with one or more injections of a polypeptide preparation, including but not limited to rabbits, mice, rats, etc.

Various adjuvants may be used to increase the immunological response in the host animal, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminium hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, oil emulsions, keyhole lympet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum.

A monoclonal antibody to an epitope of a protein may be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein (1975, Nature 256, 493-497), and the more recent human B-cell hybridoma technique (Kesber *et al.* 1983, Immunology Today 4:72) and EBV-hybridoma technique (Cole *et al.* 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc. pp. 77-96). In addition, techniques developed for the production of "chimeric antibodies" by splicing the genes from an antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity may be used (Morrison *et al.* 1984, Proc. Natl. Acad. Sci., 81:6851-6855; Neuberger *et al.* 1984 Nature 312:604-608; Takeda *et al.* 1985 Nature 31:452-454). Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce specific single chain antibodies.

Recombinant human or humanized versions of monoclonal antibodies are a preferred embodiment for human therapeutic applications. Humanized antibodies may be prepared according to procedures in the literature (e.g. Jones *et al.* 1986, Nature 321:522-25; Reichman *et al.* 1988, Nature 332:323-27; Verhoeyen *et al.* 1988, Science 239:1534-36). The recently described "gene conversion mutagenesis" strategy for the production of humanized monoclonal antibody may also be employed in the production of humanized antibodies (Carter *et al.* 1992 Proc. Natl. Acad. Sci. U.S.A. 89:4285-89). Alternatively, techniques for generating the recombinant phage library of random combinations of heavy and light regions may be used to prepare recombinant antibodies (e.g. Huse *et al.* 1989 Science 246:1275-81).

Antibody fragments which contain the idiotype of the molecule such as Fu $F(ab^1)$ and $F(ab^2)$ may be generated by known techniques. For example,

such fragments include but are not limited to: the F(ab) E2 fragment which can be produced by pepsin digestion of the intact antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the F(ab')2 fragment, and the two Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent. Alternatively, Fab expression libraries may be constructed (Huse *et al.* 1989, Science 246:1275-1281) to allow rapid and easy cloning of a monoclonal Fab fragment with the desired specificity to a protein.

Adjuvants and Carriers

Pharmaceutically acceptable carriers or diluents include those used in compositions suitable for oral, rectal, nasal, topical (including buccal and sublingual), vaginal, parenteral (including subcutaneous, intramuscular, intravenous, intradermal, intrathecal and epidural) administration. They are non-toxic to recipients at the dosages and concentrations employed. Representative examples of pharmaceutically acceptable carriers or diluents include, but are not limited to water, isotonic solutions which are preferably buffered at a physiological pH (such as phosphate-buffered saline or Tris-buffered saline) and can also contain one or more of, mannitol, lactose, trehalose, dextrose, glycerol, ethanol or polypeptides (such as human serum albumin). The compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy.

As mentioned above the composition may include an adjuvant. As will be understood an "adjuvant" means a composition comprised of one or more substances that enhances the immunogenicity and efficacy of a vaccine composition. Non-limiting examples of suitable adjuvants include squalane and squalene (or other oils of animal origin); block copolymers; detergents such as Tween®-80; Quil® A, mineral oils such as Drakeol or Marcol, vegetable oils such as peanut oil; *Corynebacterium*-derived adjuvants such as *Corynebacterium parvum*; *Propionibacterium*-derived adjuvants such as *Propionibacterium acne*; *Mycobacterium bovis* (Bacille Calmette and Guerin or BCG); interleukins such as interleukin 2 and interleukin 12; monokines such as interleukin 1; tumour necrosis factor; interferons such as gamma interferon; combinations such as saponin-aluminium hydroxide or Quil-A

aluminium hydroxide; liposomes; ISCOM adjuvant; mycobacterial cell wall extract; synthetic glycopeptides such as murarmyl dipeptides or other derivatives; Avridine; Lipid A derivatives; dextran sulfate; DEAE-Dextran or with aluminium phosphate; carboxypolymethylene such as Carbopol' EMA; acrylic copolymer emulsions such as Neocryl A640 (e.g. U.S. Pat. No. 5,047,238); vaccinia or animal poxvirus proteins; sub-viral particle adjuvants such as cholera toxin, or mixtures thereof.

Gene/DNA Isolation

The DNA encoding a protein may be obtained from any cDNA library prepared from tissue believed to express the gene mRNA and to express it at a detectable level. DNA can also be obtained from a genomic library.

Libraries are screened with probes or analytical tools designed to identify the gene of interest or the protein encoded by it. For cDNA expression libraries, suitable probes include monoclonal or polyclonal antibodies that recognize and specifically bind the protein; oligonucleotides of about 20-80 bases in length that encode known or suspected portions of cDNA from the same or different species; and/or complementary or homologous cDNAs or fragments thereof that encode the same or a hybridizing gene. Appropriate probes for screening genomic DNA libraries include, but are not limited to, oligonucleotides; cDNAs or fragments thereof that encode the same or hybridizing DNA including expressed sequence tags and the like; and/or homologous genomic DNAs or fragments thereof. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures as described in chapters 10-12 of Sambrook *et al.*

An alternative means to isolate a gene encoding the protein of interest is to use polymerase chain reaction (PCR) methodology as described in section 14 of Sambrook *et al.* This method requires the use of oligonucleotide probes that will hybridize to the gene.

The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false positives are minimized. The actual nucleotide sequence(s) is usually based on conserved or highly homologous nucleotide sequences or regions of the gene. The oligonucleotides may be degenerate at one or more positions. The use of

degenerate oligonucleotides may be of particular importance where a library is screened from a species in which preferential codon usage in that species is known. The oligonucleotide must be labelled such that it can be detected upon hybridization to DNA in the library being screened. The preferred method of labelling is to use (α-^{32}P)- dATP with polynucleotide kinase, as is well known in the art, to radiolabel the oligonucleotide. However, other methods may be used to label the oligonucleotide, including, but not limited to, biotinylation or enzyme labelling.

DNA encompassing all the protein coding sequence is obtained by screening selected cDNA or genomic libraries, and if necessary, using conventional primer extension procedures as described in section 7.79 of Sambrook *et al.*, to detect precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA.

Another alternative method for obtaining the gene of interest is to chemically synthesize it using one of the methods described in Fingels *et al.* (*Agnew Chem. Int. Ed. Engl. 28:* 716-734, 1989). These methods include triester, phosphite, phosphoramidite and H-Phosphonate methods, PCR and other autoprimer methods, and oligonucleotide syntheses on solid supports. These methods may be used if the entire nucleic acid sequence of the gene is known, or the sequence of the nucleic acid complementary to the coding strand is available, or alternatively, if the target amino acid sequence is known, one may infer potential nucleic acid sequences using known and preferred coding residues for each amino acid residue.

Substantially Purified

By "substantially purified" we mean a polypeptide that has been separated from lipids, nucleic acids, other polypeptides, and other contaminating molecules.

Preferably the isolated polynucleotide molecule encoding the FIRE polypeptide has at least 90%, more preferably 95%, identity to the nucleotide sequence shown in Figure 3, especially from nucleotide 218 to 2260

In a preferred embodiment, the polynucleotide sequence is less than 5000 nucleotides, however, it can be less than 1000 or even 500 nucleotides in length. Preferrably, the polynucleotides of the present invention are at least 18 nucleotides in length.

The polynucleotide sequence of the present invention may hybridise to the sequence set out in Figure 3 under high stringency. As used herein, stringent conditions are those that (i) employ low ionic strength and high temperature for washing after hybridization, for example, 0.1 x SSC and 0.1% (w/v) SDS at 50°C; (ii) employ during hybridization conditions such that the hybridization temperature is 25°C lower than the duplex melting temperature of the hybridizing polynucleotides, for example 1.5 x SSPE, 10% (w/v) polyethylene glycol 6000, 7% (w/v) SDS, 0.25 mg/ml fragmented herring sperm DNA at 65°C; or (iii) for example, 0.5M sodium phosphate, pH 7.2, 5mM EDTA, 7% (w/v) SDS and 0.5% (w/v) BLOTTO at 70°C; or (iv) employ during hybridization a denaturing agent such as formamide, for example, 50% (v/v) formamide with 5 x SSC, 50mM sodium phosphate (pH 6.5) and 5 x Denhardt's solution (32) at 42°C; or (v) employ, for example, 50% (v/v) formamide, 5 x SSC, 50mM sodium phosphate (pH 6.8), 0.1% (w/v) sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50μg/ml) and 10% dextran sulphate at 42°C.

Materials and Methods

1. Isolation of Dendritic cells (DC)

The procedure for the isolation of DC subpopulations has been described elsewhere in detail (Vremec *et al*, (1992). J. Exp. Med. 176: 47-58; Kronin *et al*. (1996). J. Immunol, 157; 3819). Briefly, spleens were digested with collagenase (1mg/ml; Worthington type II) and DNAase at room temperature for 20 min, followed by EDTA treatment for 5 min to disrupt DC-T cell complexes. Remaining procedures were conducted at 4°C. Low density cells were enriched by centrifugation for 10 min in Nycodenz medium (1.077 g/cm^3 mouse osmolarity). The low density cells were incubated with a mixture of mAb consisting of: anti-CD3, KT3-1.1; anti-CD4, GK1.5; anti-Th1.2, 30-H12; anti-Gr-1, RB68C5; anti-F4/80, anti-B220, RA36B2; and anti-erythrocytes, TER119. All the mAb were used at pre-titrated levels. Antibody coated cells were depleted with anti-rat IgG-conjugated magnetic beads, used at 5:1 bead-to-cell-ratio. The remaining cells were stained with fluorochrome-conjugated anti-CD11c and anti-CD8α mAb and propidium iodide (to label and exclude dead cells). Populations of > 95% pure viable $CD11c^+$ $CD8\alpha^+$ and $CD11c^+$ $CD8\alpha^-$ DC were isolated by sorting on a MoFlow

instrument (Cytomation Inc.). Cells were snap frozen and stored at -70°C until used to extract RNA.

2. RDA (Representational Difference Analysis)

RNA was extracted using QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech) and cDNA was synthesised (cDNA Synthesis Kit, Boehringer Mannheim Biochemica) according to the manufacturer's instruction. The cDNA RDA method was essentially as described by Hubank and Schatz (Nuc. Acid. Res. 22: 5640-5648, 1994). Minor alternations to this protocol include the amount of starting RNA. Due to the scarcity of the two DC populations, a total of 5 x 10^5 $CD8^-Mac\text{-}1^+$ DC and 1.8 x 10^6 $CD8^+Mac\text{-}1^-$ DC were used to extract mRNA. The synthesised double stranded cDNA was then digested with *Dpn*II and purified by phenol extraction and ethanol precipitation in the presence of 2 µg glycogen. Digested cDNA was annealed with the R-Bgl-24 and R-Bgl-12 oligonucleotides and ligated with T4 DNA Ligase (1200 units) at 14°C for 12-16 h. To compensate for the fact that three-fold more $CD8^+Mac\text{-}1^-$ DC were used to obtain mRNA, the ligated cDNA was diluted by a factor of three. Aliquots (1 µl) of the ligation mixture were amplified in multiple 100 µl polymerase chain reactions (PCR) using the R-Bgl-24 oligonucleotide. The PCR reaction contained; 66mM Tris-HCl (pH8.8), 4mM $MgCl_2$, 16mM $(NH_4)_2SO_4$; 33 µg/ml BSA, dATP, dCTP, dGTP, and dTTP (all 0.3 mM) and 2 µg R-Bgl-24 primer. The R-Bgl-12 oligonucleotide was melted away at 72°C (3 min) and the 3'ends were filled in with 5 U Taq DNA polymerase (Perkin Elmer) at 72°C (5 min). Twenty cycles of amplification were performed (1min, 95°C; 3 min, 72°C). Amplification products were visualised on a 1.3% agarose gel containing ethidium bromide which confirmed that each sample gave rise to a similar concentration of representations. Products of each representation were then combined, phenol extracted, ethanol precipitated and resuspended in TE at 0.5 µg/ml. The R-adapters were removed from the representation with *Dpn*II and the digest was phenol extracted and ethanol precipitated to form the driver. Twenty micrograms of this driver was further gel-purified on a 1.2% TAE agarose gel, and the product, which was now free of the R-adapter, was isolated using QIAEX (Qiagen). This formed the "tester" of which 2 µg were ligated to the J-Bgl-12/24 adapter in the same manner as described above. For the first subtractive hybridisation step, 0.4 µg J-ligated tester ($CD8^+$ DC) was

mixed with 40 μg of driver ($CD8^-$ DC) and *visa versa*. The mixture was phenol extracted, ethanol precipitated, and resuspended in 4 μl of EEx3 buffer (30 mM EPPS (Sigma), pH 8.0; 3 mM EDTA). The solution was overlayed with mineral oil and the DNA was denatured for 6 min (98°C). The salt concentration was adjusted with 1 μl of 5M NaCl and the sample was allowed to anneal for 20 h (67°C). The hybridised sample was diluted with 8 μl TE (10mM Tris, 1mM EDTA, pH8.0) containing 5 mg/ml yeast RNA and then resuspended in a total volume of 400 μl TE. For each subtraction, four 200 μl PCR reactions containing 20 μl of hybridisation mix were set up as previously, but the primer was omitted. Again, the 12-mer oligo (R-Bgl-12) was melted away and 3' ends were filled using Taq DNA polymerase, then 2 μg of J-24-mer was added. After ten cycles of amplification, the four reactions were pooled, phenol extracted, isopropanol precipitated, and resuspended in 40 μl of 0.2xTE. Twenty microlitres of the product was digested with 20 U of mung bean nuclease and the reaction was stopped after 30 min by the addition of 50 mM Tris-HCl (pH8.9). The digest was heated to 98°C (5 min), chilled on ice then used in the final amplification. Four PCRs were conducted per hybridisation. Each PCR containing 20 μl of MBN-treated product and 2μg J-Bgl-24 was heated to 80°C, before 5 U of Taq DNA polymerase was added and further 18 amplification cycles were performed. The four reactions were pooled, phenol extracted, isopropanol precipitated, and resuspended at 0.5 μg/μl, giving the first differential product (DP1). The J-adapters were changed with N-Bgl-12/24 adapter and the process was repeated, with the exception that 50 ng tester was mixed with 40 μg of driver (i.e. 1:800). To generate the final DP3 product, 100 pg of J-ligated DP2 was mixed with 40 μg driver (i.e. 1:400,000) and the process was repeated except that the final amplification was performed for 22 cycles (70°C, 3 min; 95°C. 1 min).

3. Protein expression of FIRE domains

3.1. Oligonucleotides

Four cDNA constructs consisting of various extracellular domains of the FIRE clones were amplified by PCR using the following oligonucleotides:
(i) FIRE EGF domain 1;
Forward primer (5'-3'): CTAC GGATCC AAT ATT TCA GCT TCC TGT CC;
Reverse primer (5'-3'): CGCG AAGCTT TCA ATC TTG ACA TTT CTC ATG G
(ii) FIRE EGF domain 2;
Forward primer(5'-3'): GACG GGATCC AAT GAG TGT CTA CTG AAA GAA TTG;
Reverse primer (5'-3'): ACCG AAGCTT TCA GCT CTT GTT CAC ATA ACA ATC.
(iii) FIRE EGF domain 1 & 2;
Forward primer (5'-3'): CTAC GGATCC AAT ATT TCA GCT TCC TGT CC;
Reverse primer (5'-3'): ACCG AAGCTT TCA GCT CTT GTT CAC ATA ACA ATC.
(iv) FIRE Hinge;
Forward primer (5'-3'): ACAC GGATCC ACT TTG GGA GTA CTG AGT GAA;
Reverse primer (5'-3'): CGCT AAGCTT TCA TAG AGC CAT GAG CAC AGC A.

3.2. PCR Protocol

The oligonucleotide pairs listed above were used to amplify the corresponding FIRE domains from BlueScript plasmid DNA (1 µl of 1:10 dilution) containing FIRE cDNA inserts.

The final concentration of each oligonucleotide in the PCR was 400 nM, magnesium concentration was 2 mM and Elongase (Gibco-BRL) was used as the polymerase in all reactions. PCR were conducted over 32 cycles as follows (cycle 1 94°C, 1 min; cycle 2-31: 94°C, 30 sec, 58°C, 30 sec (or 55°C to amplify FIRE EGF1 domain), 68°C, 30 sec; cycle 32: 68°C, 5 min)

3.3. Cloning of PCR Products

PCR products were electrophoresed through 2% agarose gels containing ethidium bromide. The bands of interest were excised and the DNA was purified from the gel pieces using a 'Qiaex II Gel Extraction Kit' (Qiagen) according to the manufacturer's recommendations. The purified DNA from each PCR contained a *Bam*HI recognition site at its 5' end (indicated by the single underlined region in the sequences of all forward primers above). This *Bam*HI recognition site (and other restriction endonuclease recognition sites described below) was 'protected' during PCR by four non-specific flanking bases, indicated by double-underlining in Section 3.1. The DNA amplified by all primers of contructs (i)-(iv) (Section 3.1) contained a *Hind*III recognition sequence at its 3' end (indicated by single underline). All PCR products also contained a stop codon at their 3' ends (encoded in the reverse primer and shown in bold in section 1). Each of the purified PCR products was then cut with the appropriate restriction enzymes; ie; Constructs (i)-(iv) were cut with *Bam*HI and *Hind*III. Similarly, two vectors that had been chosen for bacterial expression, pMalp2 (which will express the FIRE domains as fusion proteins with maltose binding protein) and pCaln (which will express the FIRE domains as a fusion with calmodulin binding protein), were treated with *Bam*HI and *Hind*III (both vectors). The PCR products were ligated into the appropriate vector and these plasmids were then used to transfect the *E.coli* strain, DH5α.

4. Expression of fusion proteins of FIRE Domains in *E. coli*

Colonies were selected for protein expression on the basis of Ampicillin resistance and the presence of an insert of the expected size. Clones containing FIRE inserts in the pMALp2 plasmid were grown up for protein expression following the recommended protocol for periplasmic protein expression (New England Biolabs, pMalp2 Expression System Manual). The FIRE/maltose binding protein fusion proteins were purified over a 5 ml Amylose column (New England BioLabs) which was eluted with 15 x 0.5 ml aliquots of 20 mM Tris [(pH 8.0, HCl), 200 mM NaCl, 1 mM EDTA] containing 1 mM maltose. A small sample of the eluates was examined by 12% SDS-PAGE for the presence of protein of the expected size.

Protein- containing eluates were pooled and dialysed against mouse tonicity PBS. Clones containing FIRE inserts in the pCaln plasmid were grown up for protein expression following the recommendations of the manufacturer (Affinity Protein Expression and Purification System, Stratagene). Fusion proteins were purified over a 10 ml calmodulin column (Stratagene) which was eluted with 20 x 0.5 ml aliquots of PBS containing 2 mM EDTA. A small sample of the eluates was examined by 16% Tricine SDS-PAGE for the presence of protein of the expected size. Protein-containing eluates were pooled and dialysed against mouse tonicity PBS.

5. Elicitation of Antisera

Wistar rats of approximately 10 weeks old were injected with the FIRE fusion proteins. Specifically, pre-bleeds were taken by cardiac puncture (approximately 1 ml of blood was obtained from each rat) and the following day the rats were injected with an emulsion of fusion protein in Complete Freunds Adjuvant (CFA). Approximately 200 µg of each fusion protein was given per rat on the first day of injection. The route of injection was subcutaneous, normally in two sites. Three weeks after the first injection, the rats were injected with the same fusion protein in CFA, using the same route of injection. Preliminary data indicates that antibodies which can recognise recombinant FIRE EGF domains on western blots have already been elicited.

6. Expression in Eukaryotic Cells

Transfection of eukaryotic cells such as CHO and COS-1 cells will be attempted using standard transfection reagents such as FuGENE-6 (Boehringer Mannheim) according to the manufacturer's recommendations. Briefly, 1-2 µg of plasmid DNA containing full length sequence of FIRE will be mixed with FuGENE-6 Transfection reagent at a ratio of 1:6, then added to the monolayer of CHO/COS-1 cells or 293T cells. Incubation time will depend upon time required to express the reporter gene of interest. Selection for neomycin resistance using G418 is also possible using this protocol. Attempts will also be made to express FIRE domains as fusion proteins expressed in eukaryotic cells. Here PCR will be used to isolate cDNA encoding domains or segments of FIRE which will then be cloned into

appropriate expression vectors in a manner analagous to that described above. Thus FIRE (or segments thereof) can be expressed as a fusion protein with the Fc reigon of Immunoglobulin, or as a fusion protein with domains 3 and 4 of CD4 (Brown et al. European Journal of Immunology 25: 3222-8 1995). Alternatively, FIRE can be expressed as a fusion with a short polypeptide encoding a well characterised monoclonal antibody epitope such as OX-8 (Classon et al. International Immunology 4, 1204-1210 1992) or FLAG (Hopp et al. Biotechnology 6, 1204-1210 1978).

7. Isolation of cDNA Encoding a Partial Fragment of Human FIRE

cDNA encoding a partial fragment of human FIRE was isolated using the Polymerase Chain Reaction. Briefly, cDNA was synthesised conventionally from RNA isolated from both enriched human thymic DC populations and also from normal and activated human splenocytes. The resulting cDNA was then used as a template in a PCR using the following primers which were designed from mouse FIRE sequences: CACCTGCAGCTCTTCCATCT and GAAAGTTTGCTTCTCAAAATCCA. To allow for variation between mouse and human sequences, low stringency conditions were used for amplifiction with a Mg Cl_2 concentration of 2.5 mM and an annealing temperature of 50°.

A cDNA encoding a partial polypeptide fragment of 134 amino acids was isolated. The cDNA sequence is set out in Figure 5 and the putative amino acid sequence is set out in Figure 6. This fragment shows 75% identity to the mouse sequence at the protein level.

RESULTS

Figure 1 shows an RDA analysis comparing gene expression in the myeloid $CD8^-Mac\text{-}1^+$DC and the lymphoid $CD8^+Mac\text{-}1^-$DC. It can be seen that as successive rounds of PCR and subtractive hybridisation take place genes in common between the two populations are subtracted and not amplified (hence the background smear disappears as one goes from DP-1 (the first differential product) to DP-3 (the third differential product)). The bands which are observed in the DP-3 which correspond to fragments of putative differentially expressed cDNA molecule were cloned and sequenced. House-keeping genes were detected only at very low frequency in these

sequenced fragments suggesting that the RDA efficiently removed "common" sequences. To confirm that the bands from the DP-3 products were indeed differentially expressed in a minority population such as DC, several different approaches were taken. First, we generated a new "representation" from a separate source of RNA - this assures that any bias introduced in the first representation (used to generate the first RDA) would not be introduced in the reanalysis of differential expression. Using this approach, which is referred to as a "Virtual Northern", 9/11 gene fragments were found to be differentially expressed. This is a surprisingly high efficiency considering techniques such as RDA are prone to generating false positives, and indicates that this particular RDA successfully amplified differentially expressed genes. One of the DNA fragments was chosen for further analysis as it encoded a novel membrane protein, termed FIRE. Conventional Northern blot analysis (Figure 2) confirmed data from Virtual Northerns and RT-PCR that FIRE is expressed at higher levels in myeloid DC than lymphoid DC.

FIRE

Full length clones encoding this novel gene were obtained using conventional molecular biological techniques, involving the RDA generated fragments. The full length sequence of FIRE, showing both cDNA sequences and translated protein sequences, are shown in Figure 3.

FIRE encodes a 723 amino acid protein and analyses of the FIRE sequence shows that it is a novel member of the recently described EGF/TM7 superfamily (7), a comparison of the FIRE sequence with its distant relatives, the other members of the EGF/TM7 superfamily are shown in Figure 4. The most famous member of the EGF/TM7 superfamily is F4/80 (mouse EMR-1) a molecule which is a marker of macrophages. Analysis of the FIRE sequence predicts a structure where there are two EGF domains in its extracellular region together with a "hinge" structure possessing a number of putative glycosylation sites. The protein then crosses the cell membrane seven times before a sizeable cytoplasmic domain of some 94 amino acids.

FIRE is a marker for DC populations. The data obtained to date demonstrates that this molecule is expressed differentially or at much higher levels on the more "stimulatory" $CD8^-$ myeloid-related rather than the $CD8^+$ lymphoid-related dendritic cell populations, as they occur in the mouse

spleen. Accordingly, it is believed that specific ligands such as monoclonal antibodies directed to this molecule will be useful reagents in identifying and particularly in purifying dendritic cells.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Dated this ninth day of September 1999

CSL Limited, The Council of the Queensland Institute of Medical Research, The Walter and Eliza Hall Institute of Medical Research, The University of Melbourne, and Commonwealth Scientific and Industrial Research Organisation
Patent Attorneys for the Applicants:

F B RICE & CO

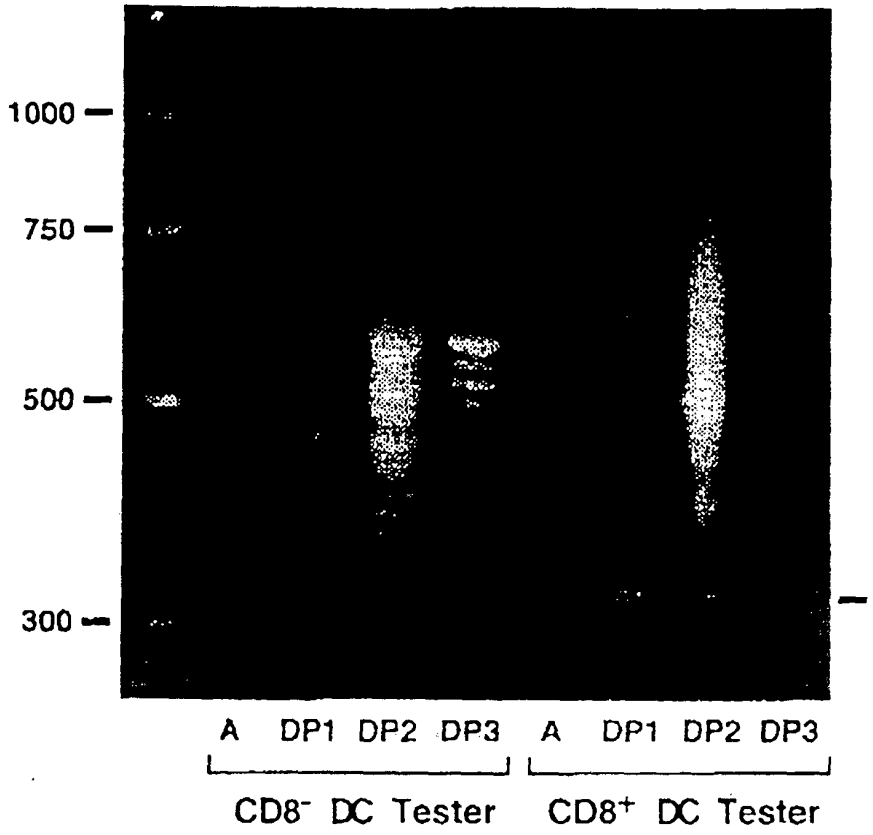

Figure 1. Representational difference analysis of DC subpopulations. The initial representation, referred to as the amplicon (A), was generated from cDNA obtained from CD8α- Mac-1+ DC (CD8-) and CD8α+ Mac-1- DC (CD8+). The cDNA from each DC population was then used as the tester from which the cDNA of the second population was subtracted. The first, second, and third subtraction and amplification cycles gave rise to the differential product (DP) 1, 2, and 3, respectively.

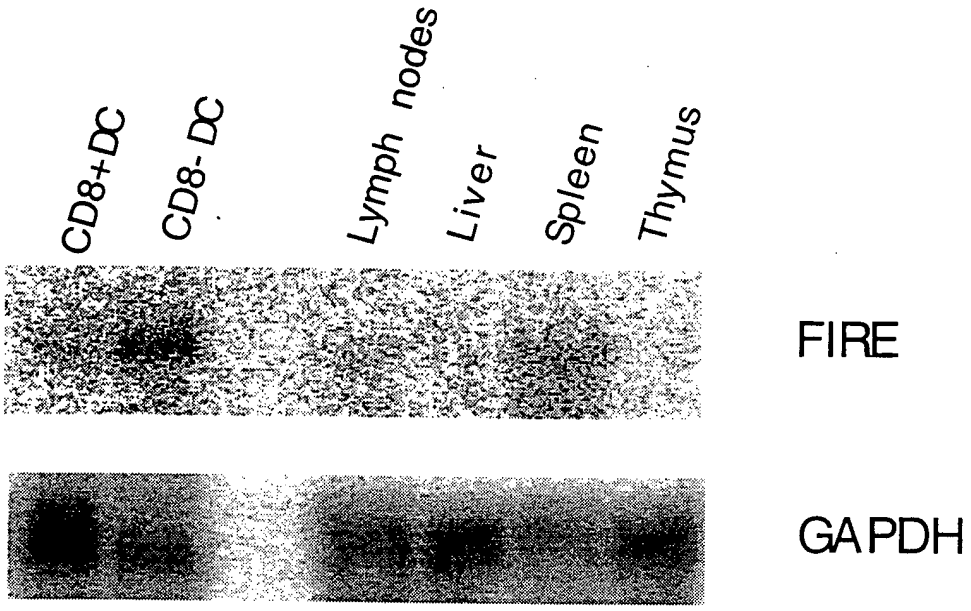

Figure 2. Northern blot analysis of FIRE. RNA was obtained from various organs and purified DC populations. After electrophoresis and transfer, the membrane was hybridised to a fragment of the FIRE cDNA. FIRE is clearly differentially expressed in the CD8- DC population, and is also weakly expressed in spleen.

Figure 3/1

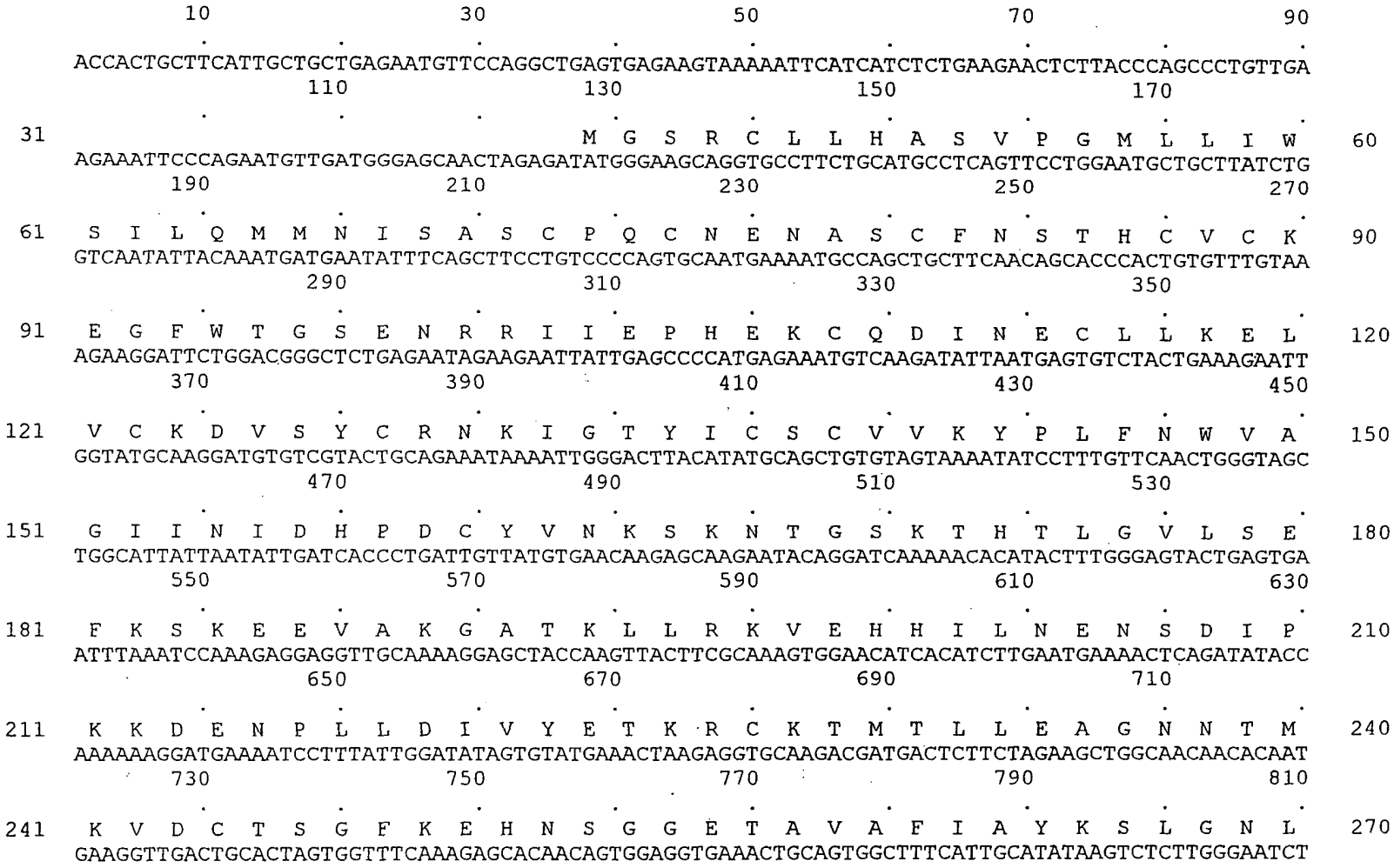

Figure 3/2

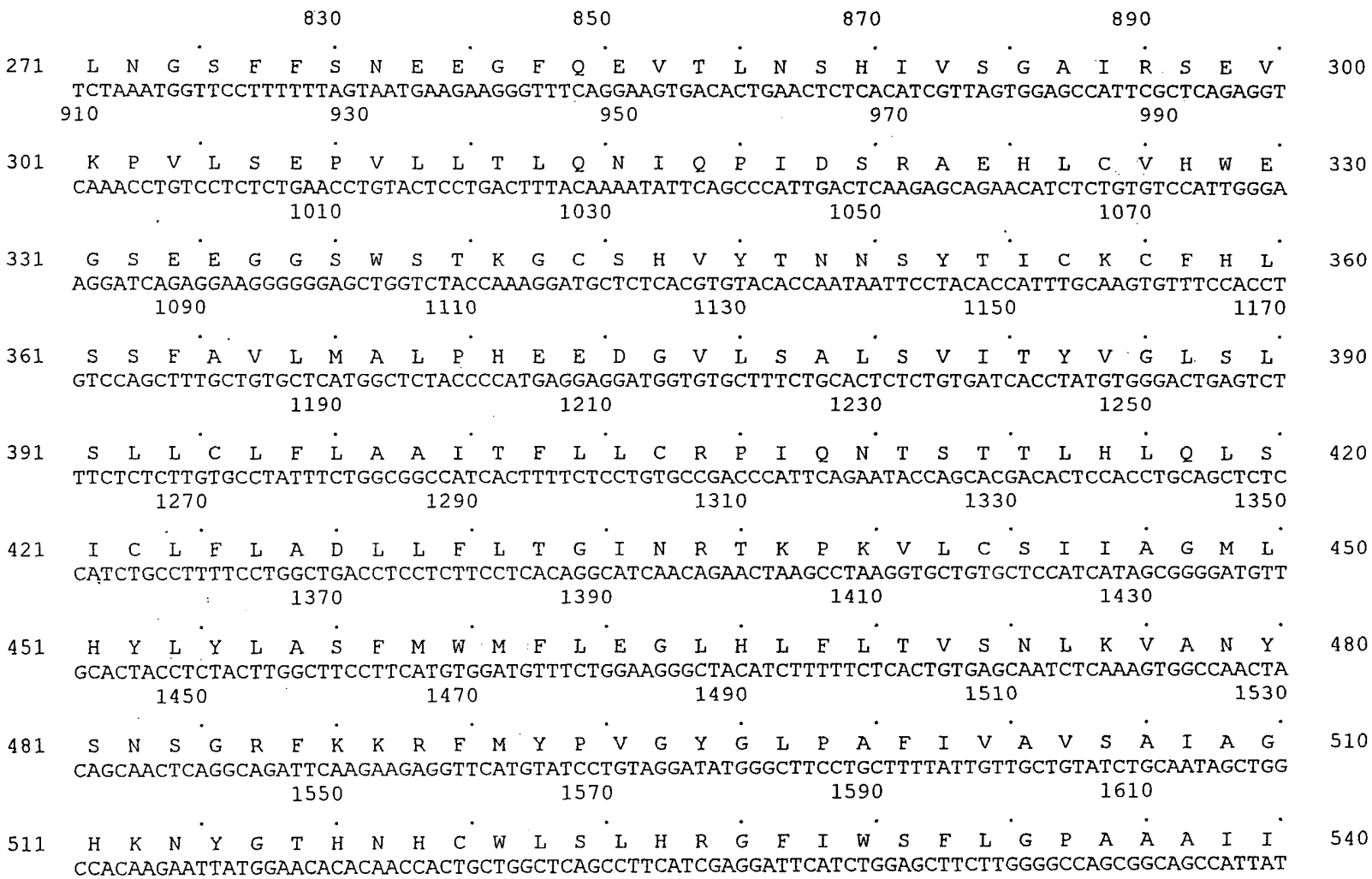
830 850 870 890
271 L N G S F F S N E E G F Q E V T L N S H I V S G A I R S E V 300
TCTAAATGGTTCCTTTTTTAGTAATGAAGAAGGGTTTCAGGAAGTGACACTGAACTCTCACATCGTTAGTGGAGCCATTCGCTCAGAGGT
910 930 950 970 990
301 K P V L S E P V L L T L Q N I Q P I D S R A E H L C V H W E 330
CAAACCTGTCCTCTCTGAACCTGTACTCCTGACTTTACAAAATATTCAGCCCATTGACTCAAGAGCAGAACATCTCTGTGTCCATTGGGA
1010 1030 1050 1070
331 G S E E G G S W S T K G C S H V Y T N N S Y T I C K C F H L 360
AGGATCAGAGGAAGGGGGGAGCTGGTCTACCAAAGGATGCTCTCACGTGTACACCAATAATTCCTACACCATTTGCAAGTGTTTCCACCT
1090 1110 1130 1150 1170
361 S S F A V L M A L P H E E D G V L S A L S V I T Y V G L S L 390
GTCCAGCTTTGCTGTGCTCATGGCTCTACCCCATGAGGAGGATGGTGTGCTTTCTGCACTCTCTGTGATCACCTATGTGGGACTGAGTCT
1190 1210 1230 1250
391 S L L C L F L A A I T F L L C R P I Q N T S T T L H L Q L S 420
TTCTCTCTTGTGCCTATTTCTGGCGGCCATCACTTTTCTCCTGTGCCGACCCATTCAGAATACCAGCACGACACTCCACCTGCAGCTCTC
1270 1290 1310 1330 1350
421 I C L F L A D L L F L T G I N R T K P K V L C S I I A G M L 450
CATCTGCCTTTTCCTGGCTGACCTCCTCTTCCTCACAGGCATCAACAGAACTAAGCCTAAGGTGCTGTGCTCCATCATAGCGGGGATGTT
1370 1390 1410 1430
451 H Y L Y L A S F M W M F L E G L H L F L T V S N L K V A N Y 480
GCACTACCTCTACTTGGCTTCCTTCATGTGGATGTTTCTGGAAGGGCTACATCTTTTTCTCACTGTGAGCAATCTCAAAGTGGCCAACTA
1450 1470 1490 1510 1530
481 S N S G R F K K R F M Y P V G Y G L P A F I V A V S A I A G 510
CAGCAACTCAGGCAGATTCAAGAAGAGGTTCATGTATCCTGTAGGATATGGGCTTCCTGCTTTTATTGTTGCTGTATCTGCAATAGCTGG
1550 1570 1590 1610
511 H K N Y G T H N H C W L S L H R G F I W S F L G P A A A I I 540
CCACAAGAATTATGGAACACACAACCACTGCTGGCTCAGCCTTCATCGAGGATTCATCTGGAGCTTCTTGGGGCCAGCGGCAGCCATTAT

Figure 3/3

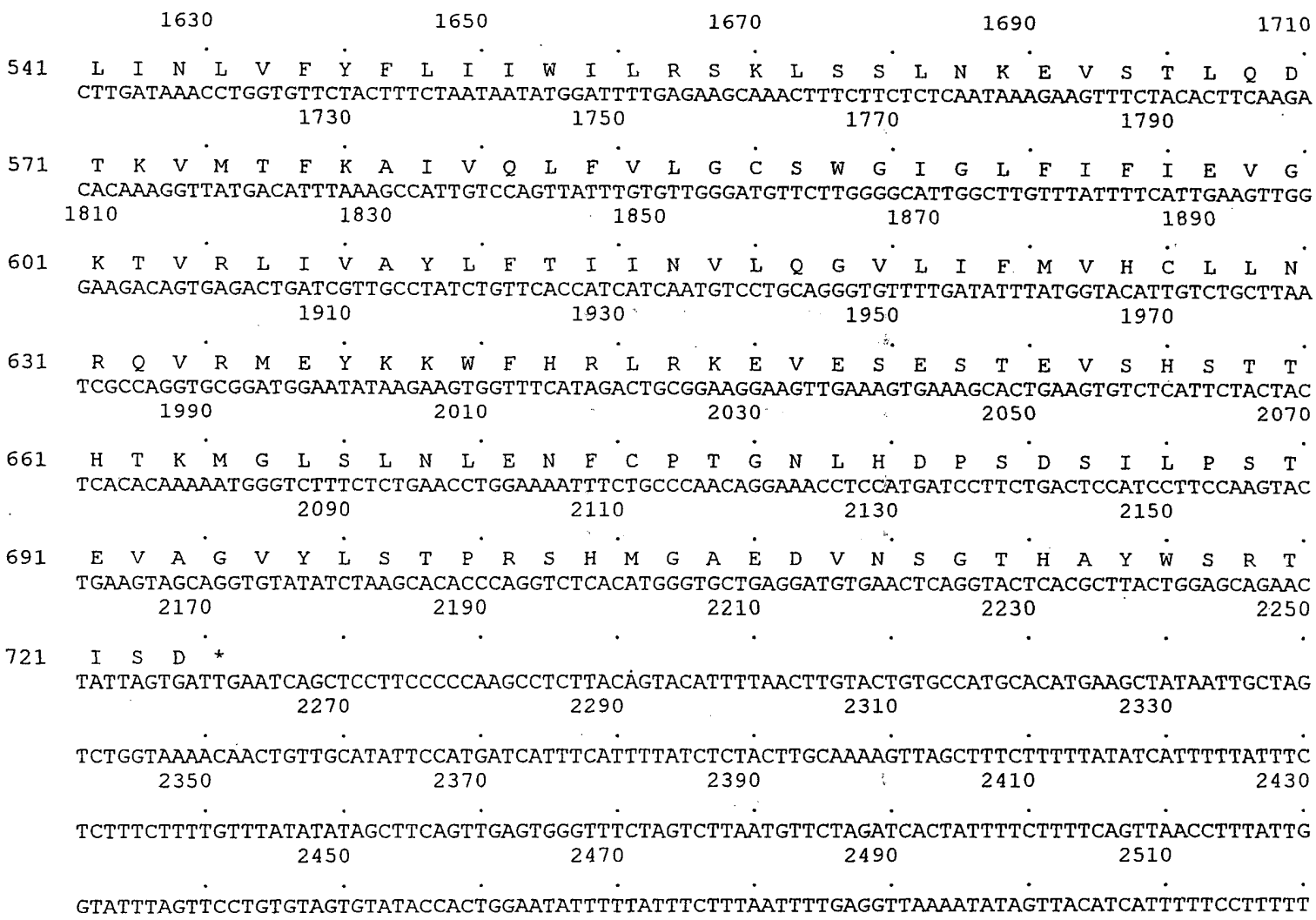

Figure 3/4

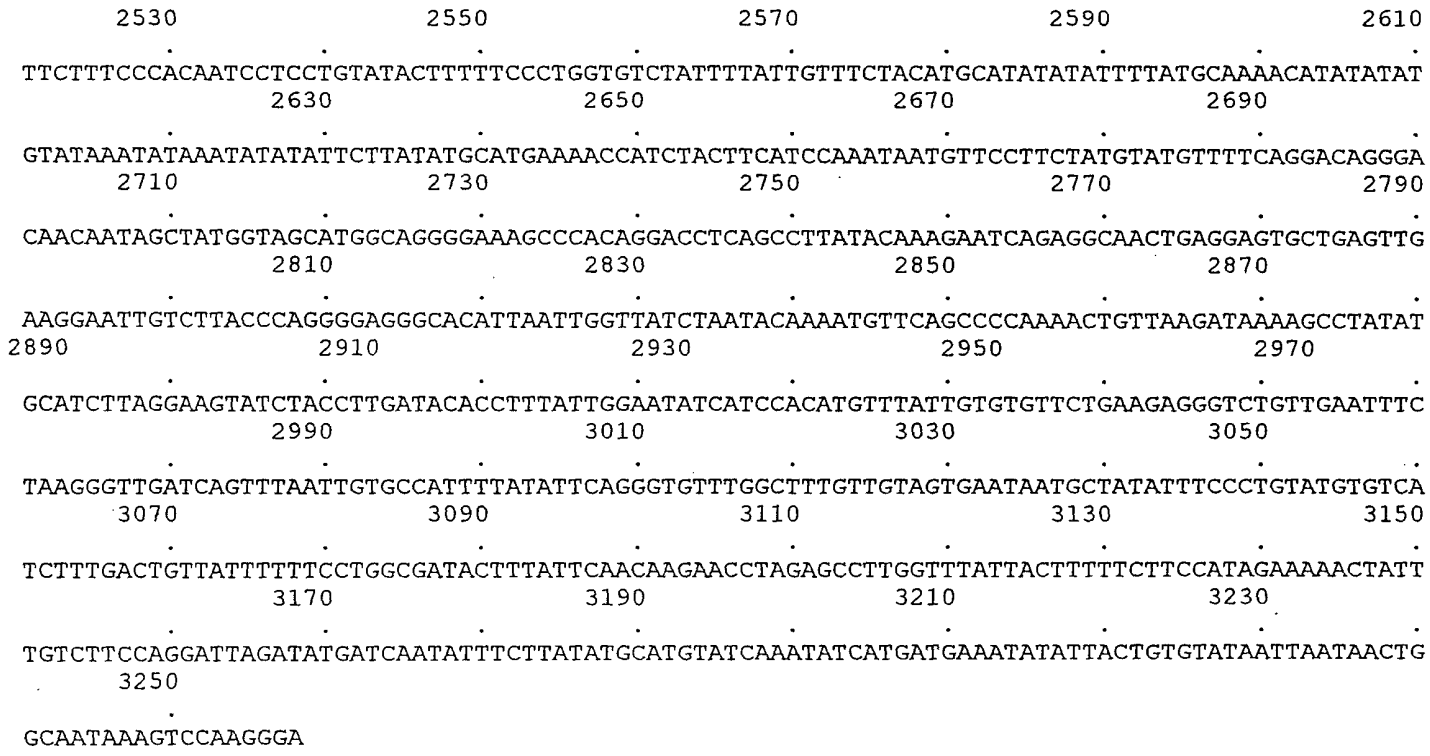

FIG. 4/1

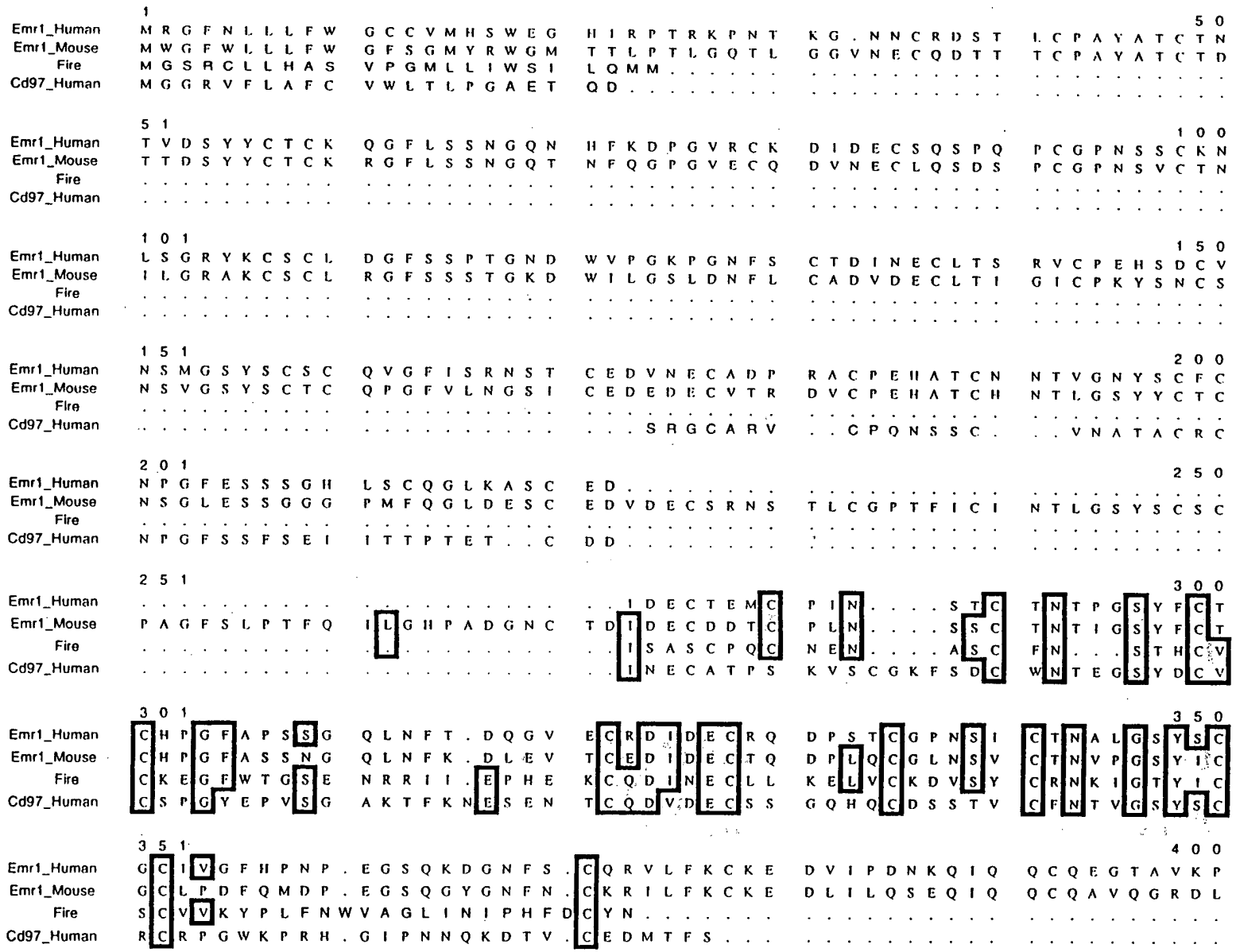

FIG 4/2

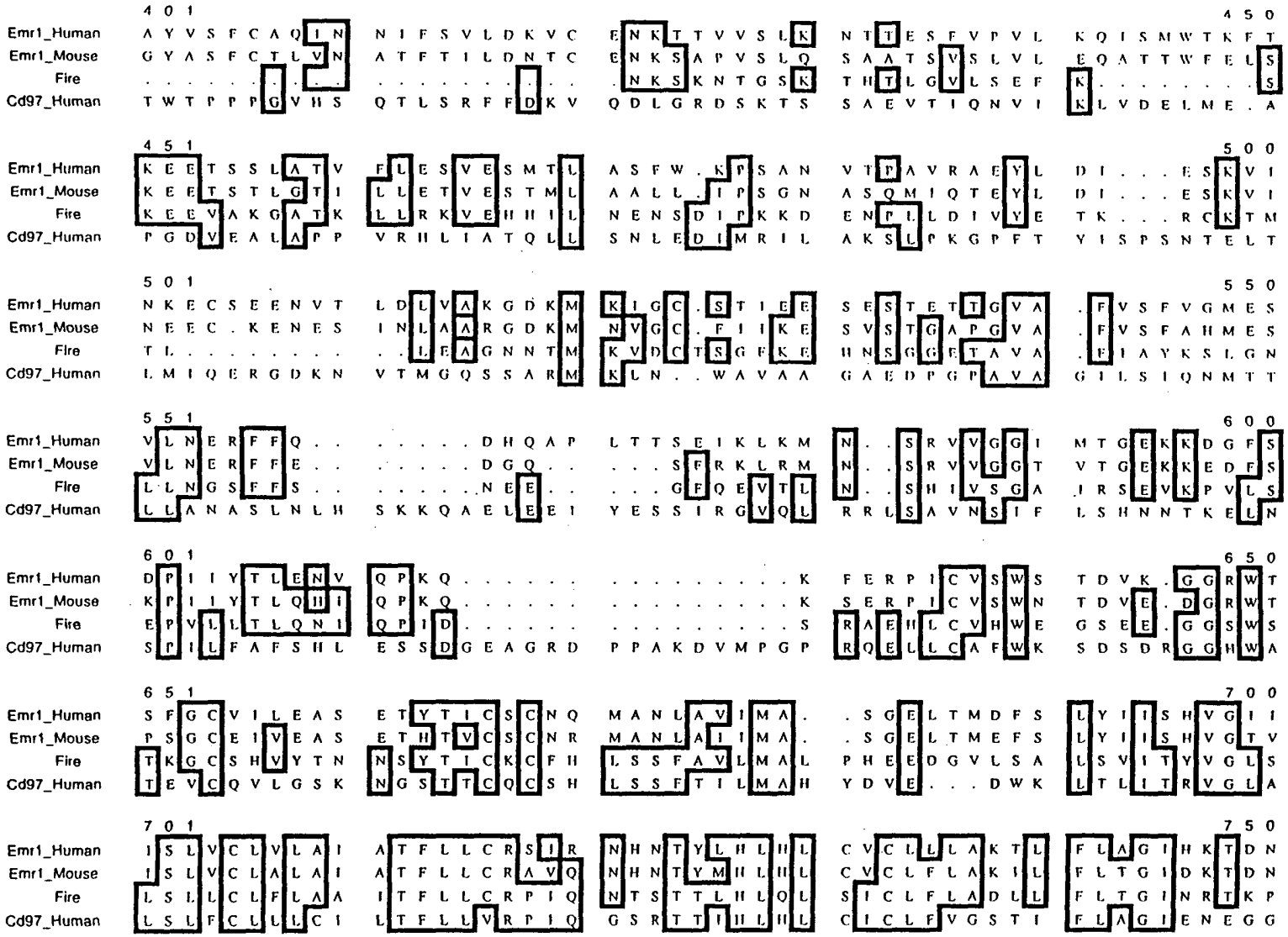
401 450
Emr1_Human AYVSFCAQIN NIFSVLDKVC ENKTTVVSLK NTTESFVPVL KQISMWTKFT
Emr1_Mouse GYASFCTLVN ATFTILDNTC ENKSAPVSLQ SAATSVSLVL EQATTWFELS
Fire .... NKSKNTGSK THTLGVLSEF K.....S
Cd97_Human TWTPPPGVHS QTLSRFFDKV QDLGRDSKTS SAEVTIQNVI KLVDELME.A
451 500
Emr1_Human KEETSSLATV FLESVESMTL ASFW.KPSAN VTPAVRAEYL DI...ESKVI
Emr1_Mouse KEETSTLGTI LLETVESTML AALL.IPSGN ASQMIQTEYL DI...ESKVI
Fire KEEVAKGATK LLRKVEHHIL NENSDIPKKD ENPLLDIVYE TK...RCKTM
Cd97_Human PGDVEALAPP VRHLIATQLL SNLEDIMRIL AKSLPKGPFT YISPSNTELT
501 550
Emr1_Human NKECSEENVT LDLVAKGDKM KIGC.STIEE SESTETTGVA .FVSFVGMES
Emr1_Mouse NEEC.KENES INLAARGDKM NVGC.FIIKE SVSTGAPGVA .FVSFAHMES
Fire TL..... ..LEAGNNTM KVDCTSGFKE HNSGGETAVA .FIAYKSLGN
Cd97_Human LMIQERGDKN VTMGQSSARM KLN..WAVAA GAEDPGPAVA GILSIQNMTT
551 600
Emr1_Human VLNERFFQ.. .....DHQAP LTTSEIKLKM N..SRVVGGI MTGEKKDGFS
Emr1_Mouse VLNERFFE.. .....DGQ.. ...SFRKLRM N..SRVVGGT VTGEKKEDFS
Fire LLNGSFFS.. .....NEE.. ...GFQEVTL N..SHIVSGA IRSEVKPVLS
Cd97_Human LLANASLNLH SKKQAELEEI YESSIRGVQL RRLSAVNSIF LSHNNTKELN
601 650
Emr1_Human DPIIYTLENV QPKQ.....K FERPICVSWS TDVK.GGRWT
Emr1_Mouse KPIIYTLQHI QPKQ.....K SERPICVSWN TDVE.DGRWT
Fire EPVLLTLQNI QPID.....S RAEHLCVHWE GSEE.GGSWS
Cd97_Human SPILFAFSHL ESSDGEAGRD PPAKDVMPGP RQELLCAFWK SDSDRGGHWA
651 700
Emr1_Human SFGCVILEAS ETYTICSCNQ MANLAVIMA. .SGELTMDFS LYIISHVGII
Emr1_Mouse PSGCEIVEAS ETHTVCSCNR MANLAIIMA. .SGELTMEFS LYIISHVGTV
Fire TKGCSHVYTN NSYTICKCFH LSSFAVLMAL PHEEDGVLSA LSVITYVGLS
Cd97_Human TEVCQVLGSK NGSTTCQCSH LSSFTILMAH YDVE...DWK LTLITRVGLA
701 750
Emr1_Human ISLVCLVLAI ATFLLCRSIR NHNTYLHLHL CVCLLLAKTL FLAGIHKTDN
Emr1_Mouse ISLVCLALAI ATFLLCRAVQ NHNTYMHLHL CVCLFLAKIL FLTGIDKTDN
Fire LSLLCLFLAA ITFLLCRPIQ NTSTTLHLQL SICLFLADLL FLTGINRTKP
Cd97_Human LSLFCLLLCI LTFLLVRPIQ GSRTTIHLHL CICLFVGSTI FLAGIENEGG

FIG 4/3

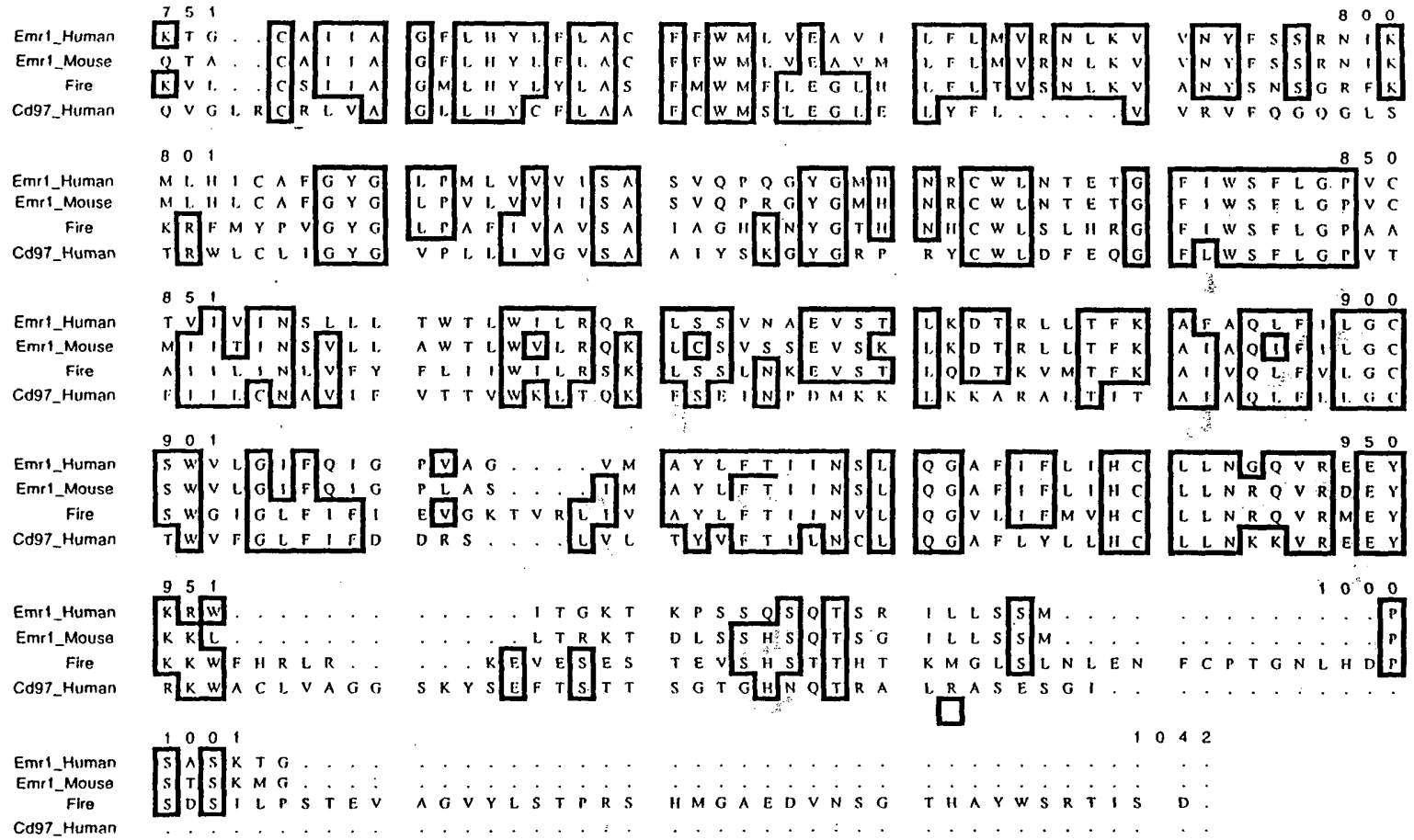

Figure 5

ELSLCLFLAHLLFLTGINRTEPEVLCSIIAGLLHFLYLACFTWMLLEGLH
LFLTVRNLKVANYTSTGRFKKRFMYPVGYGIPAVIIAVSAIVGPQNYGTF
THCWLKLDKGFIWSFMGPVAVIILINLVFYFQVL